Glutathione Peroxidase 8 is transcriptionally regulated by HIFα and modulates growth factor signaling in HeLa cells

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ABSTRACT

GPx8 is a mammalian Cys-glutathione peroxidase of the endoplasmic reticulum membrane, involved in protein folding. Its regulation is mostly unknown. We addressed both, functionality of two hypoxia response elements (HREs) within the promoter, *GPx8*-HRE1 and *GPx8*-HRE2 and the GPx8 physiological role. In HeLa cells, treatment with HIFα stabilizers, such as diethyl succinate (DES) or 2-2'–bipyridyl (BP) induces GPx8 mRNA 1.5 fold. Luciferase activity of pGL3^{GPx8wt}, containing a fragment of the GPx8 promoter including the two HREs, is also induced by DES/BP or by overexpressing either individual HIFα subunit. Mutating *GPx8*-HRE1 within pGL3^{GPx8wt} resulted in a significantly higher inhibition of luciferase activity than mutating *GPx8*-HRE2. EMSA analysis showed that both HREs exhibit enhanced binding to a nuclear extract from DES/BP-treated cells, with stronger binding by *GPx8*-HRE1. In DES-treated cells transfected with pGL3^{GPx8wt} or mutants thereof, silencing of HIF2α, but not HIF1α, abolishes luciferase activity. Thus *GPx8* is a novel HIF target preferentially responding to HIF2α binding at its two novel functional *GPx8*-HREs, with *GPx8*-HRE1 playing the major role. FGF treatment increases GPx8 mRNA expression and reporter gene experiments indicate that induction occurs via HIF. Comparing the effect of depleting GPx8 on the downstream effectors of FGF or insulin signaling, revealed that absence of GPx8 results in a 16 or 12 fold increase of phosphorylated ERK 1/2 - by FGF or insulin treatment respectively. Furthermore, in GPx8 depleted cells, phosphorylation of AKT by insulin treatment increases 2.5 fold. We suggest that induction of GPx8 expression by HIF slows down proliferative signaling during hypoxia and/or growth stimulation through receptor tyrosine kinases.

Keywords: GPx8; Endoplasmic reticulum; ER; Hydroperoxide; Hypoxia; Receptor Tyrosine kinase; RTK; Redox; Signaling

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Introduction

In excess, hydroperoxides (ROOH)[1] have a pro-inflammatory role and are toxic to cells. However, when produced in limited amounts associated with physiological signaling, ROOH appear to modulate redox sensitive processes including growth, differentiation, and proliferation [1-4]. Emerging knowledge suggests that ROOH are required for receptor tyrosine kinase (RTK) signaling with the intriguing function of amplifying the RTK signaling cascades [1,5].

The glutathione peroxidase (GPx) family of proteins encompasses distinct gene products that efficiently reduce ROOH into corresponding alcohols. Inverse genetic studies suggest that they are non-redundant enzymes, despite catalyzing a similar reaction and often exhibiting an overlapping cellular distribution [6]. In humans, the family of glutathione peroxidases includes members that may contain either the rare amino acid selenocysteine (Sec), or the more common cysteine (Cys) as the redox active moiety. Apart from that, in both subfamilies the redox-active residue is included in a conserved catalytic tetrad (Fig. 1) [7].

In humans, the Sec subfamily (SecGPx) is comprised of four tetrameric peroxidases (GPx1, GPx2, GPx3, and GPx6) that use GSH to reduce H_2O_2 and other small ROOH, including free fatty acid hydroperoxides, and one monomeric peroxidase, GPx4 that exhibits unusual preferences toward both the oxidizing and the reducing substrates. Indeed, GPx4 efficiently reduces, in addition to small ROOH that are substrate for the tetrameric SecGPx, phospholipid and cholesterol or cholesterol ester hydroperoxides incorporated in membranes or lipoproteins [8]. Consequently, unique among the SecGPx, GPx4 is a vital enzyme preventing ferroptotic cell death *in vivo*, where lipid peroxidation is involved [9,10]. Nevertheless, GPx4 also accepts protein thiols as electron donors when GSH is limiting, a property related to its role in male fertility [11,12]. However, recent research suggests that all the SecGPx are relevant players in inflammation, cancer, proliferation, signaling as reviewed in [13]. As a whole, the importance of the SecGPx subfamily has contributed to the perception of a multifaceted role of ROOH in cells.

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[1] Abbreviations: AKT, protein kinase B; BP, 2,2' –bipyridyl; CysGPx, glutathione peroxidase containing cysteine as the redox-active moiety; DES, diethyl succinate; EPO, erythropoietin; ER, endoplasmic reticulum; ERK1/2, extracellular regulated kinases 1 and 2; ERO-1, ER, oxidoreductin 1; FGF, fibroblast growth factor –acidic; GPx, glutathione peroxidase; GPx8, glutathione peroxidase family member 8; HIF, hypoxia inducible factor; h*GPx8*, gene encoding human glutathione peroxidase 8, HRE, hypoxia response elements; c-JNK, Jun NH(2)-terminal kinase; MAPK, mitogen-activated protein kinase, NOX4, NADPH oxidase family member 4; PLOOH, phospholipid hydroperoxide; PTEN, tensin homolog deleted on chromosome 10; PTP, protein tyrosine phosphatase; PTP1B, PTP family member 1B; ROOH, hydroperoxide; P-AKT, phosphorylated protein kinase B; PGK, phosphoglycerate kinase; PDI, protein disulfide isomerase; P-ERK1/2, phosphorylated extracellular regulated kinases 1 and 2; PKA, protein kinase A; Prx4, peroxiredoxin 4; TK, thymidine kinase; SecGPx, glutathione peroxidase containing selenocysteine as the redox-active moiety; RTK, receptor tyrosine kinase; PTEN, tensin homolog deleted on chromosome 10

The Cys subfamily (CysGPx) is comprised, in humans, of three members that have been much less studied than the SecGPxs. Structurally, two are monomeric proteins, namely GPx7 and GPx8 [14,15] and one, GPx5, is a tetrameric enzyme specifically secreted from the epididymis [16] (Figure 1). GPx7 and GPx8 are the last discovered members and thus among the most mysterious in terms of function. Evolutionary studies suggested that they derived from the SecGPx4, which actually had a Cys-containing ancestor. Thus, human GPx7 and GPx8 represent a recent return to Cys usage in glutathione peroxidases, which is not easily rationalized [17].

Overall, steady-state kinetic studies of various Cys variants of glutathione peroxidases indicate that the step reducing ROOH is not dramatically affected by replacing Sec by Cys. This contrasts with the reducing step by glutathione regenerating the ground state enzyme, which is much slower and severely affects the enzyme turnover [7,18]. Yet, the artificially constructed CysGPx4 can rescue the death phenotype of SecGPx4 lacking cells [19] demonstrating that a Cys to Sec replacement does not prevent the vital functions of the enzyme, at least in cells.

GPx7 and GPx8 are widely distributed in mammals. Unique among the glutathione peroxidases family members, they both contain an endoplasmic reticulum (ER) retention signal at the protein C-terminal end (Fig. 1). GPx7 is free in the lumen while GPx8 is an intrinsic membrane peroxidase with its active site facing the lumen [15,20]. Peculiarly, they may accept protein disulfide isomerase (PDI) as a reductant more efficiently than GSH [15,18,21].

The ER location and reactivity with PDI prompted the proposal that GPx7 and GPx8 are involved in oxidative protein folding. This apparently occurs by re-oxidizing PDI in the peroxidatic reaction, where H_2O_2, produced by ER oxidoreductin (ERO-1), is the oxidant [15,21]. Peroxiredoxin 4 (Prx4), a peroxidase present in the ER in most tissues as a secretory protein [22], is apparently much less efficient in removing ER H_2O_2 compared with GPx7 and GPx8 [15,21,23]. Furthermore, kinetic analysis shows that recombinant GPx7 may reduce both, H_2O_2 or phospholipid hydroperoxide (PLOOH) by both, GSH or PDI, where kinetic constant measurements and concentration of the two reductants within the ER comply with the proposal that the actual GSH concentration within the ER might modulate PDI oxidation [18]. On the other hand, whether reduction of PLOOH is a functional role in GPx7 physiology remains to be addressed.

GPx7 has been described as a tumor suppressor. Together with human *GPx3*, the human *GPx7* promoter was found hyper-methylated and thus down-regulated in some premalignant lesions of the esophagus [24]. Furthermore, in the epithelial cells of the esophagus, GPx7 could protect against oxidative damage of DNA and regulate oxidative signals that depend on the mitogen activated protein kinases (MAPK) p38MAPK and c-Jun NH(2)-terminal kinase (JNK) upon exposure to pH 4 and bile acids [25]. More recently, it was documented that loss of GPx7 resulted in systemic oxidative

damage, shortened life span and increased carcinogenesis in mice [26]. Furthermore, GPx7 deficiency has been linked to obesity and pre-adipocyte differentiation [27] by controlling the dimerization of protein kinase A (PKA) and activating the CCAAT/enhancer binding protein beta.

Compared to GPx7, knowledge on GPx8 is much more limited. The enzyme has not been fully characterized kinetically, although it seems less efficiently reduced by PDI than GPx7 [15]. Apparently, however, GPx8 efficiently prevents the spillover of H_2O_2 generated from the ER by ERO-1 [23]. GPx8 was described as one of the cellular substrate of the Hepatitis C virus NS3-4A protease [20] and, according to a transcriptomic and proteomic profiling of KEAP1, it is down-regulated in breast epithelial sulforaphane-treated cells, which suggests that Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) indirectly dampens GPx8 expression [28].

The studies presented here were designed to address the physiological function of *GPx*8 by examining the functionality of two Hypoxia Inducible Factor (HIF) binding sites in the promoter. Indeed little is known about the link between HIF and glutathione peroxidases. Only the human plasma glutathione peroxidase (GPx3) was described to contain a binding site for HIF-1 and it is indeed induced by hypoxia [29]. Similarly the expression of the human GPx1 was found linked to oxygen sensing, but through the action of two oxygen responsive promoter elements (ORE) [30]. ORE is distinct from hypoxia response element (HRE) and apparently responds to a milder hypoxia [31]. Notably, however the HIF subunits are not only stabilized by hypoxia, but by signaling as well, where most likely common triggers are reactive nitrogen and oxygen species [32]. We discovered that *GPx8* is indeed a HIF target and, as such, up-regulated by chemical hypoxia and fibroblast growth factor (FGF) treatment. Furthermore, GPx8 depletion in cells affected FGF and insulin signaling. All together these findings link the expression of *GPx8* to HIF stabilization, and expand its function to the control of RTK signaling cascades.

Material and Methods

Cell culture and treatments

HeLa cells (ATCC CCL-2^{TM}) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine (Life Technologies). Treatments were performed on 70 % confluent cells after overnight starvation in the absence of FBS. To stabilize HIFα subunits, 20 mM diethyl succinate (DES) or 0.1 mM 2,2' –bipyridyl (BP) (Sigma) was added and cells further incubated in serum-free medium for approximately 16-h. In some experiments, 100 ng/ml of recombinant human fibroblast growth factor – acidic (FGF) (Life technology), was used in serum-free medium for 30 min and cells collected after 24-h in complete medium.

GPx8 silenced HeLa cells contained a vector for stable expression of small interfering RNA addressed to GPx8 (SilenciX technology) and were purchased from Tebu-bio, which also provided HeLa cells transfected with a control shRNA. The percentage of silencing was 97%, as quantified by the manufacturer. GPx8 silenced cells and controls thereof were grown as above except that the medium contained 4 mM L- glutamine, 110 mg/l sodium pyruvate and 125 µg/ml hygromycin B (Life Technologies). These were treated with 100 ng/ml of FGF or 20 µg/ml insulin for 10 min in serum free medium and lysed as described below. Proteins were quantified by the Bradford reagent (Sigma).

mRNA analysis and quantification

RNA was extracted by TRIzol reagent (Life Technology) and retrotranscribed using the Taqman reverse transcription reagent (Applied Biosystem). Retrotranscribed RNA (100 ng) was amplified using the QuantumRNA Classic II 18S (Life Technology) in the presence of 5% dimethyl sulfoxide and 1 µM of the *Hs*GPx8-fw and *Hs*GPx8-rev primers (Table 1). Normalization was achieved by measuring 18S RNA. The annealing temperature was 59 °C. A Kodak image station was used for band quantification. Relative expression was calculated as the ratio between sample and 18 S RNA.

Constructs

The pGL3^{PGKHRE} containing six repetitions of the human phosphoglycerate kinase (PGK) HREs upstream the thymidine kinase (TK) promoter is described in [33]. The pGL3-based luciferase

reporter pGL3^{GPx8wt}, which contains the two putative *GPx8*-HRE1 and *GPx8*-HRE2 in a pGL3Basic vector (Promega) was obtained by cloning a 1373 bp fragment (from nt -1391 to -18) of the human *GPx8* promoter into the KpnI and BglII sites of the pGL3Basic vector. The fragment was obtained by PCR from the genomic DNA obtained from white blood cells using the pGL3^{GPx8wt}-fw and pGL3^{GPx8wt}-rev primers (Table 1).

The constructs carrying mutations at the *GPx8*-HRE1 or *GPx8*-HRE2 or both the cassettes (pGL3^{GPx8mut1}, pGL3^{GPx8mut2}, and pGL3$^{GPx8mut1-2}$ respectively), were generated by PCR using Quick change II according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA) using the pGL3^{GPx8mut1}-fw and pGL3^{GPx8mut1}-rev or pGL3^{GPx8mut2}-fw and pGL3^{GPx8mut2}-rev nucleotides respectively (Table 1). In pGL3^{GPx8mut1}, the *GPx8*- HRE1 wild type 'ACGTG' cassette is exchanged with 'TTAAT'. Similarly, in the pGL3^{GPx8mut2}, the *GPx8*-HRE2 wild type 'GCGTG' cassette is exchanged with 'TTAAT'. The pGL3$^{GPx8mut1-2}$ contains both, *GPx8*-HRE1 and *GPx8*-HRE2, exchanged with the 'TTAAT' sequence and was obtained by subsequent mutation of the *GPx8*-HRE2 cassette of the pGL3^{GPx8mut1} by the above technique with the primers pGL3^{GPx8mut2}-fw and pGL3^{GPx8mut2}- rev (Table 1). Constructs accuracy was verified by sequencing.

Cell transfection and reporter gene experiments

HeLa cells were seeded onto 24-well plates, grown to 60 % confluence, and transfected with 0.2 µg of the experimental constructs and the same amount of pRL-TK vector expressing *Renilla* luciferase (Promega) for control of transfection efficiency- as described in [34], except that TransIT-LT1 (Mirus) was used as transfection reagent. Firefly luciferase activity of the experimental constructs was normalized to the *Renilla* luciferase activity obtained with the control vector pRL-TK and expressed as fold increase of luciferase activity. This is the ratio between the normalized luminescence observed in the treated sample and that in the control sample, which was treated with vehicle. In the experiments where transient expression of human HIF1α subunits was used, the cotransfection mixture contained, beside the appropriate experimental construct and pRL-TK vector, 0.2 µg of a construct expressing stable HIF1α or HIF2α [33] and were collected after 48-hr.

In the experiments where the HIFα subunits were silenced, HeLa cells were first transfected with the oligonucleotides SiRNA-HIF1α–sense or SiRNA-HIF2α–sense (Table 1) and corresponding antisense, for HIF1α or HIF2α silencing, respectively, by the TransIT –siQuest transfection reagent (Mirus). Controls were produced by transfecting SiRNA-scramble-sense and corresponding antisense (Table 1). After four hours, cells were transfected with the experimental luciferase reporter construct as described above, and 20mM DES was used to stabilize HIFα subunits. The

effectiveness of siRNA treatment was verified by PCR on extracted and retrotranscribed RNA (see above) from silenced HeLa cells using the primers *Hs*HIF1α–fw and *Hs*HIF1α–rev or *Hs*HIF2α–fw and *Hs*HIF2α–rev (Table 1).

Nuclear extracts preparation and EMSA analysis

HeLa cells were treated with DES or BP or vehicle as above, and the nuclear extract were prepared according to [35], except that cells were collected in hypotonic buffer and immediately frozen in liquid nitrogen before starting the nuclear preparation. Before use, the nuclear extract was dialyzed by a 8 kDa cutoff mini dialysis kit, (GE Healthcare Biosciences).

EMSA analysis was performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific) and used according to the manufacturer's instructions. Biotinylated oligonucleotide probes of identical length were used. These contained the *GPx8*-HRE1 and *GPx8*-HRE2. As a positive control a biotinylated probe of the same length containing four repetitions of EPO-HREs was used. (*GPx8*-HRE1-fw, *GPx8*-HRE1-rev; *GPx8*-HRE2-fw, *GPx8*-HRE2-rev, *EPO*-HREs-fw, *EPO*-HREs-rev in Table 1). Nuclear extract (7 µg of proteins) was used for the binding reaction. When present, competitor DNAs were pre-incubated with the nuclear extract before adding the biotinylated probe. Electrophoresis was performed on 6 % non-denaturing polyacrylamide gels (DNA retardation gels, Life Technologies)

Preparation of cellular extracts, immunoblot analysis and quantification

After treatments, HeLa cells, GPx8 - silenced HeLa cells and controls were lysed by 0.1 M Tris-HCl (pH 7.5), 150 mM KCl, 1 mM EDTA, 0.5 % Triton X-100 and 1 % protease inhibitors (Protease Inhibitor Cocktail, Sigma) and phosphatases Inhibitor (Phosphatase Inhibitor Cocktail 2, Sigma). The whole extract, containing approximately 20 to 40 µg of protein, was dissolved in Laemmli buffer containing 1 M 2-mercaptoethanol, denatured at 95 ^{0}C and resolved on a NuPage 4–12 % BisTris mini gel (Life Technologies) and transferred to a nitrocellulose membrane using a buffer containing 25 mM ethanolamine, 104 mM glycine, 20 % methanol, pH 9.5. After an overnight blotting at 180 mA, the nitrocellulose membrane was appropriately cut and immunoblotted with specific antibodies against GPx8 (Abnova), PDI (BD Transduction Laboratories), phosphorylated extracellular signal regulated kinase 1/2 (P-ERK1/2, pT42 and pY44), (Santa Cruz Biotechnology) or phosphorylated protein kinase B (P-AKT, pS473) (Cell Signaling Technology) or anti HSP90 α/β (Santa Cruz Biotechnology).

For ERK1/2 detection, the blotting probed with P-ERK was stripped and re-probed with antibodies against ERK1/2 (Santa Cruz Biotechnology). Immunoreactive bands were detected using appropriate secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) followed by chemiluminescence detection on a Kodak Image station.

Results

The promoter of GPx8 contains two putative Hypoxia Response Elements (HREs)

In 2010, the promoters of all the glutathione peroxidases were screened by an optimized analysis method for transcription factor binding sites, which matched different searching programs, i.e. Match, P-Match, MatInspector [36]. The promoter of GPx8 emerged as a possible HIF target since two conserved HREs were predicted by comparing various *GPx8* sequences from different mammals. Supported by the observation that, in the human gene, these two putative HREs (*GPx8*-HRE1 and *GPx8*-HRE2, Fig. 2) exactly match the HIF- DNA binding motif (5'-RCGTG-3') described in [32], we recently decided to address whether the human *GPx8* is a HIF target and whether the predicted *GPx8*-HRE1 and *GPx8*-HRE2 are functional sites in the human gene (h*GPx8*).

hGPx8 mRNA and protein are induced by HIF alpha stabilization

We first addressed whether, in cultured cells, the amount of GPx8 mRNA is increased when HIF alpha subunits are stabilized. To this end, HeLa cells were treated with BP or DES, which stabilize HIFα subunits by HIF prolyl 4-hydroxylase inhibition [37,38]. Fig. 3 shows that both GPx8 mRNA and protein are significantly increased following cell treatment with either 100 µM BP or 20 mM DES, indicating that HIFα stabilization results in increased GPx8 expression.

Both the HREs within the hGPx8 are functional

Next, we addressed whether the fragment of the promoter containing the putative *GPx8*-HRE1 and *GPx8*-HRE2 could support GPx8 transcriptional activation, and whether the two HREs are involved. To this purpose reporter gene experiments were conducted. First, $pGL3^{GPx8wt}$, a luciferase reporter containing a fragment of 1373 nt upstream the transcriptional start of *GPx8* (from -18 to -1391), containing the two putative HREs, was used for transfecting HeLa cells. Following a treatment with DES or BP, luciferase activity was measured. As a positive control, cells were transfected with a luciferase reporter containing six repetitions of the PGK HRE upstream the TK promoter ($PGL3^{PGKHRE}$) [33], a well - known HIFα target.

As shown in Fig. 4A both, DES or BP treatment increased luciferase activity five or four times respectively in $pGL3^{GPx8wt}$ - transfected cells. An increase of approximately two or three times was also observed respectively in both, DES or BP-treated cells transfected with $pGL3^{PGKHRE}$. In cells transfected with $pGL3^{GPx8wt}$ or $pGL3^{PGKHRE}$ luciferase activity was also increased by co-transfection

with stable HIF1α or HIF2α (Figure 4B). We concluded that the examined fragment of the h*GPx8* promoter could drive transcription following HIF alpha subunits stabilization. Next, an experiment similar to that in Fig. 4A was conducted, but plasmids carrying individual mutation at the *GPx8*-HRE1 or *GPx8*-HRE2, or at the two sites simultaneously, pGL3^{GPx8mut1}, pGL3$^{GPx8\ mut2}$ and pGL3$^{GPx8\ mut1-2}$ respectively, were also used (Fig. 5). Luciferase activity dropped from 30 to 70 % in the DES - or BP - treated cells containing the mutated constructs. Notably however, the cells transfected with the pGL3^{GPx8mut1}, containing the *GPx8*-HRE2 site only, exhibited a significant higher inhibition when compared to those transfected with pGL3^{GPx8mut2}, containing *GPx8*-HRE1 only, suggesting a major role of the *GPx8*-HRE1 site in promoting expression. In the cells transfected with the double mutated construct pGL3$^{GPx8\ mut1-2}$, luciferase activity in response to DES or BP was not completely inhibited, indicating that some flanking elements may have a role in the HIF-driven expression of GPx8 or that DES or BP may cause some HIF-independent luciferase expression. This was not further investigated. Collectively, these experiments reveal that both *GPx8*-HRE1 and *GPx8*-HRE2 are novel functional sites in HeLa cells, *GPx8*-HRE1 having a major role.

Nuclear extracts containing stabilized HIFα exhibit binding activity with GPx8-HRE1 and GPx8-HRE2

Whether the two HREs directly bind to stabilized nuclear HIF was addressed by gel retardation assay. Two distinct synthetic fragments encompassing respectively the *GPx8*-HRE1 and *GPx8*-HRE2 were incubated with nuclei obtained from DES or BP treated cells and subjected to EMSA. A synthetic fragment encompassing the HRE of erythropoietin (*EPO*-HRE) of identical length was used as a positive control. EPO is indeed a well known HIF target [33]. DES or BP treated nuclei yielded an enhanced DNA binding activity to both the HREs (Figure 6 A and B, lanes 3, 4). Similarly, DES-treated nuclei yielded enhanced DNA binding to *EPO*-HRE (Figure 6 C lane 3), suggesting therefore that stable HIF alpha is indeed the protein involved. This however could not be further verified by super-shift, since a suitable anti-HIF antibody for this purpose was not found. However, the observation that all protein - fragments binding complexes migrated identically, strongly suggested that HIF alpha is present in the complex (Figure 6 D).

Interestingly, the binding of HIFα to *GPx8*-HRE1 appears stronger than that to *GPx8*-HRE2, as a higher concentration of unlabeled probe was required to decrease the DNA binding activity in the competition experiment (Figure 6 A, B, lanes 5 and 6). Thus apparently both, *GPx8*-HRE1 and *GPx8*-HRE2, directly bind to stable HIF alpha, the *GPx8*-HRE-1 site displaying higher affinity.

The two HREs of GPx8 are preferentially HIF2α targets

SiRNA technology was used to address which of the two HIFα subunits targets the HREs of GPx8. Cells were silenced by siRNA directed to HIF1α or HIF2α, co-transfected respectively with pGL3^{GPx8wt}, pGL3^{GPx8mut1}, pGL3$^{GPx8\ mut2}$ and treated with DES. In control cells, scrambled siRNA was used for co-transfection. Luciferase activity measurements showed that increase of luciferase activity after DES treatment is almost completely abrogated after HIF2α but not HIF1α silencing (Fig. 7). Thus apparently both *GPx8*-HRE1 and *GPx8*-HRE2 are preferentially HIF2α targets.

GPx8 resides in the ER where it is involved in oxidative protein folding by oxidizing PDI and GSH [15,18,23]. However, our observation that GPx8 is among the plethora of HIFα - activated genes that have critical roles in cell survival, proliferation, metabolism [39], together with the observation that the ER is a critical player in signaling [40], prompted to investigate whether the peroxidase could have a role in growth factors signaling. Because HIF1α synthesis is increased by signaling from receptor tyrosine kinases (RTK) by the MAP kinase (MPK) pathways [32], we addressed whether FGF treatment can increase GPx8 expression.

FGF increases GPx8 expression

Results in Fig. 8A shows that GPx8 mRNA is increased by FGF treatment in HeLa cells. In agreement, under these conditions, cells bearing the reporter construct pGL3^{GPx8wt} exhibit increased luciferase activity following FGF treatment. A similar increase is also observed in the control cells transfected with pGL3^{PGKHRE} (Fig. 8B), suggesting that, in our conditions, HIF is involved in the increased expression of GPx8 by FGF. On these bases we hypothesized that GPx8 might have a role in controlling FGF signaling and investigated this possibility

Loss of GPx8 up-regulates FGF and insulin signaling

To address the impact of GPx8 in FGF signaling, we used HeLa cells stably expressing a SiRNA targeted to GPx8 and controls thereof and we measured the extent of phosphorylation of ERK1/2 following FGF treatment. To extend the observation to RTK signaling, we also focused on the insulin signaling cascade.

Indeed silencing of GPx8 increased phosphorylation of the MAP kinases ERK1/2 induced by FGF sixteen fold, suggesting that the peroxidase negatively regulates FGF signaling. Furthermore, GPx8 silenced cells responded to insulin treatment with an increased ERK1/2 and AKT phosphorylation

(12 and 2.7 fold respectively) (Figure 9 A and B). We concluded that, in HeLa cells, GPx8 is a master regulator of FGF and insulin signaling.

Discussion

This study reveals that HIF alpha subunits stabilizers, such as DES or BP that inhibit iron-dependent prolyl 4-hydroxylases by competitive inhibition or iron chelation respectively, increase expression of GPx8, one of the mammalian Cys glutathione peroxidases of the ER. These treatments also increase the activity of a luciferase reporter driven by a fragment of the *GPx8* promoter encompassing the two putative HREs ($pGL3^{GPx8wt}$), which are equally increased by overexpression of stable HIF1α and HIF2α subunits. Similarly, activity of a luciferase reporter containing repetitions of the HRE site of phosphoglycerate kinase ($pGL3^{PGKHRE}$), an established HIF target, is increased. Between the two sites, *GPx8*-HRE1 appears to have the dominant role. Mutating the *GPx8*-HRE1 within $pGL3^{GPx8wt}$ produces a more pronounced drop of luciferase activity than does mutating the *GPx8*-HRE2. Furthermore, as demonstrated in a competitive gel retardation assay, inhibition of binding of *GPx8*-HRE1 to a nuclear extract containing stabilized HIF requires a greater amount of an unlabeled HIFα fragment, than does *GPx8*-HRE2, indicating a higher affinity of *GPx8*-HRE1 to HIFα.
Collectively these results demonstrate that *GPx8* is a novel HIFα target and, by binding HIF and promoting transcription, *GPx8*-HRE1 and *GPx8*-HRE2 are novel functional sites. Yet, they are not identical, being endowed with individual activity and affinity.
Despite incomplete knowledge of how mammalian glutathione peroxidases are regulated, we know that, at least one other glutathione peroxidase is a HIF target. Indeed, GPx3, a SecGPx resident in plasma and extracellular fluids, contains one HIF binding site and is strongly up-regulated by hypoxia in renal cell lines [29]. Interestingly, both GPx3 and GPx8 are linked to the ER, the former as a peroxidase that travels this compartment in secretory cells, and the latter as a resident protein of the ER membrane. However, not all of the ER glutathione peroxidase genes are HIF targets. Human *GPx7*, which express the other CysGPx located in the soluble compartment of the ER, does not contain conserved HREs and, accordingly, does not respond to HIF stabilizers (M. Maiorino, unpublished), an observation highlighting that glutathione peroxidases are part of networks subjected to distinct control mechanisms.
HIFs are $\alpha\beta$ heterodimers, HIF1α and HIF2α being constitutively expressed and oxygen-regulated at the protein level and endowed of distinct roles [32,33,38,41]. In DES-treated cells, luciferase activity of the $pGL3^{GPx8wt}$ or of the plasmids carrying mutation at HREs ($pGL3^{GPx8mut1}$ or $pGL3^{GPx8mut2}$) is almost completely abrogated when the HIF2α, but not the HIF1α subunit, is silenced (Figure 7). Apparently, therefore, both the HREs of *GPx8* are preferentially HIF2α targets. So far this finding does not conflict with the observed increased luciferase activity in cells containing the reporter $pGL3^{GPx8wt}$ upon HIF1α overexpression (Fig. 4B). It was indeed already observed that target gene

specificity can be overcome by forced expression of the HIF subunits [33] and it is not a surprise that overexpression overcomes affinity. GPx8 emerges as a preferential target of HIF2α, which drives the chronic response to hypoxia, opposite to HIF1α driving the initial response [41], but it may well be a target of HIF1α when concentration of the latter increases.

HIF is not only stabilized by hypoxia, apparently HIFα degradation is blocked by reactive oxygen and nitrogen species and HIF1α synthesis is increased by increased signaling from RTK via mitogen activated protein kinase (MAPK) pathway [32]. The latter observations comply with our finding that *GPx8* is transcriptionally activated by FGF, a cytokine acting on a RTK, which activates the downstream MAPK ERK1/2 (Figure 7A). In this experiment, specific HIF involvement is deduced because luciferase activity of both the reporters $pGL3^{GPx8wt}$ and $pGL3^{PGKHRE}$ that respond to chemical HIF stabilizers or HIF subunits overexpression (see above and Fig. 4), increases following FGF treatment (Fig. 8B).

Thus, as a HIF-target gene *GPx8* is among the plethora of genes activated by the cytokines acting on MAPK pathway, such as FGF. Whether other growth factors are involved in inducing GPx8 via HIF appears plausible, although it was not addressed in this study.

Our attempt to elucidate the functional relationship between GPx8 and FGF or agonists that activate RTK revealed that GPx8 is indeed involved in down-regulating these signaling cascades.

In GPx8-depleted HeLa cells, FGF treatment increases ERK1/2 phosphorylation, and similarly insulin treatment increases phosphorylation of both ERK1/2 and AKT (Fig. 9). As an ER peroxidase, GPx8 is perhaps in a privileged location to control the flux of H_2O_2 that specifically inactivates the protein tyrosine phosphatase (PTP) family member PTP1B [42]. Indeed PTP1B, which is apparently located in the outer membrane of the ER [40,43], has been proposed to control the extent of phosphorylation of both, RTK and insulin receptor substrate 1 (IRS-1) [44], and thus the phosphorylation status of both the downstream targets ERK and AKT that we find hyper-phosphorylated by insulin/FGF treatment in GPx8-depleted cells.

The notion that glutathione peroxidases are involved in controlling signaling cascades by RTK, that produce phosphatase-inactivating H_2O_2 via NADPH oxidase 4 (NOX4) and subsequent O_2^- dismutation [40], is emerging. Insulin-treated muscles from $GPx1^{-/-}$ mice show increased AKT phosphorylation that, remarkably is not associated with increased phosphorylation of ERK1/2 [5]. Therefore GPx1 depletion affects the redox status of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is a protein located mainly in cytosol and the nucleus [45] instead of that of the ER-residing PTP1B [5]. It emerges therefore that the phosphatase targets are distinct for the ER peroxidase GPx8 or the cytosolic/mitochondrial GPx1. Our experiments brings into focus that co-localization in the ER of NOX4, GPx8 and the phosphatase target, together with the

diffusible nature of H_2O_2, are of crucial importance in controlling signaling descending from PTP1B. This implies that the other ER peroxidases might also have the role of down-regulating PTP1B-dependent signaling as GPx8 expression does. Experimental evidence thus far seems to support this view. In esophageal cells, GPx7 was shown to regulate oxidative signals that depend on p38MAPK and JNK upon exposure to pH 4 and bile acids [25]. Furthermore, transient silencing of GPx7 in HeLa cells increases ERK1/2 phosphorylation similarly to depletion of GPx8 (V. Bosello-Travain, unpublished). Perhaps also the tumor suppressor activity in prostate cancer cells of the secretory SecGPx3 [46] might be explained by such a mechanism. On the other hand, silencing of the cytosolic GPx4 does not affect ERK1/2 signaling [47]. Furthermore, the observation that GPx1 overexpression prevented ERK1/2 phosphorylation after preconditioning neonatal mice brain with non lethal hypoxia [48], does not contradict this view, but rather suggests that overexpression allows more of GPx1 to be closer to PTP1B on the cytosolic surface of the ER.

From the viewpoint that hypoxia is associated with enhanced RTK-mediated signaling, which contributes to oncogenesis and more aggressive disease [49-51], the induction of GPx8 expression by HIF may be considered as an attempt to slow down an excessive proliferative signal during oxygen deprivation and/or RTK signaling. Under these conditions, a fine-tuning of growth and metabolism is seemingly necessary. This is apparently achieved by the interplay between positive and negative effectors [52], and, apparently, GPx8 plays the latter role.

Whether data presented here are involved with the function of GPx8 in oxidative protein folding remains to be established. It is worth to mention, however, that among the hundreds of genes that are induced by HIFα there is also ERO-1 [53], which produces H_2O_2 for GPx8. The dual role of GPx8 in oxidative folding and signaling may suggest a link between the two processes. This relationship awaits further research.

Acknowledgments

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Figure legends

Fig. 1 Multiple alignment of the eight members of the human GPx family and GPx3 from yeast, as an example of the non-vertebrate enzymes. Alignment has been obtained by clustal (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and manually edited. Amino acids whose identity threshold is above 80% are shaded in gray (the darker the most conserved). Triangles show the catalytic tetrad. Tetramer and dimer interfaces for protein oligomerization are indicated. The Cys block, containing the resolving Cys of the non-vertebrate and plant members is also indicated. The N-terminal signal peptide and the C-terminal ER retention sequence are boxed in the ER resident GPxs.

Fig. 2. Location and sequence of the putative hypoxia response elements (HRE) within the promoter of human GPx8 (h*GPx8*). A 1400 nucleotides fragment upstream the h*GPx8* ATG translation containing the two putative HREs is shown. These are located at -1298 and - 898 from the ATG. They precisely fit the consensus sequence -RCGTG- for HIF α [32], R is A in GPx8- HRE1 and G in GPx8-HRE-2.

Fig. 3. HIFα subunits stabilization increases *GPx8* mRNA (A) and protein (B) expression. HeLa cells were treated in triplicate respectively with 20 mM DES or 0.1 mM BP, for 18 hours, to stabilize HIFα subunits. Control cells were treated with vehicle (V). In (A) RNA was extracted, retrotranscribed and probed by PCR, as described in Methods. The internal control was 18S RNA. Expected length of the fragment of GPx8 is 516 bp, that of 18S RNA 320 bp. Band intensity of GPx8 was normalized to 18S RNA and fold increase of GPx8 mRNA was calculated as the ratio between normalized GPx8 expression in experimental sample vs control (vehicle). One of five independent experiments with similar results is reported. In (B) cells were lysed in the presence of protease inhibitors and 20 µg of the protein extract was subjected to SDS-PAGE followed by Western blot, which was decorated overnight by anti-GPx8 (αGPx8) or, for 1 hour, by anti-PDI (αPDI) antibodies. Immunoreactive bands were detected and quantified (see Methods for details). The intensity of the GPx8 band was normalized to the that of PDI, and fold increase of GPx8 protein was calculated as above. Significance by Student's t statistic: P<0.001 DES/BP vs. vehicle treated cells for both mRNA and protein analysis.

Fig. 4. The wild type h*GPx8* promoter activity increases by HIFα stabilization or individual HIFα subunits transfection. Promoter activity was measured by reporter gene analysis. In A) HeLa cells were transfected with the luciferase reporter construct pGL3^{GPx8wt} containing 1373 nt upstream the transcriptional start of GPx8 encompassing the two putative HREs (Fig. 1) or, for comparison, pGL3^{PGKHRE}, containing six repetitions of PGK HRE upstream the TK promoter [33]. Cells were then treated with DES or BP for 28 hours, to stabilize HIFα subunits, or vehicle as the control. In B) cells were transfected with the luciferase reporters as above, but a construct expressing stable HIF1α or HIF2α was also co-transfected and cells collected after 48 hr. Luciferase activity was normalized for transfection efficiency as indicated in Methods and fold increase was calculated as the ratio between sample and control.

Fig. 5. Mutation of each of the two GPx8-HREs decreases promoter activity. Promoter activity was measured by reporter gene analysis. HeLa cells were transfected with the wild type luciferase reporter containing the *GPx8* promoter (pGL3^{GPx8wt}) or the mutated ones carrying mutation at the *GPx8*-HRE1 or *GPx8*-HRE2 (pGL3^{GPx8mut1}, pGL3$^{GPx8\ mut2}$ respectively), pGL3$^{GPx8\ mut1\text{-}2}$ carrying mutation at both sites, and treated with DES or BP. Controls were treated with vehicle. The percentage of inhibition was calculated taking the fold increase of luciferase activity of DES/BP-treated cells transfected with the control plasmid (pGL3^{GPx8wt}) as 100%, thus the percentage of inhibition of luciferase activity is 0 in these cells. Actual value $\pm$ SD is 5.06 $\pm$ 0.63 or 3.96 $\pm$ 0.9024 in the presence of DES or BP respectively. Significance by Student's t statistic: P< 0.05 pGL3^{GPx8mut1} vs. pGL3$^{GPx8\ mut2}$ treated with DES; P<0.001 pGL3^{GPx8mut1} vs. pGL3$^{GPx8\ mut2}$ treated with BP.

Fig. 6. Nuclear extracts containing stabilized HIFα subunits exhibits DNA binding activity when incubated with *GPx8*-HRE1 or *GPx8*-HRE2. Gel retardation assays were performed by biotin labeled double stranded oligonucleotides (DSO) as indicated on top. Oligonucleotides were incubated with a nuclear extract from HeLa cells previously treated with DES or BP (20 mM or 0.1 mM respectively, for 18 hours) to stabilize the HIFα subunits or vehicle as a control. Competition was performed with the indicated molar excess of the unlabeled specific oligonucleotide that was added during the binding procedure, as a specificity control. Oligonucleotides were as follows: A) *GPx8*-HRE1, B) *GPx8*-HRE2, C) EPO-HRE. All of the oligonucleotides were of similar length and, in D the binding of the indicated fragments to the nuclear extract is compared on the same gel. Lanes are

as follows: 1, DSO; 2, DSO, incubated with a nuclear extract from vehicle treated cells (control); 3, DSO incubated with a nuclear extract from DES-treated cells; 4, DSO incubated with a nuclear extract from BP-treated cells. 5,6,7 competition with unlabeled DSO at the indicated molar concentration; a, c, f, DSO, incubated with a nuclear extract from vehicle treated cells (control); b, d, g DSO incubated with a nuclear extract from DES-treated cells; e, GPx8-HRE2 DSO.

Fig. 7. Silencing of the individual HIFα subunits indicate that HIF2α targets both the *GPx8*-HREs. Promoter activity was measured by reporter gene analysis. HeLa cells were transfected with the indicated siRNA and, four hours later, with the reporter constructs pGL3^{GPx8wt}, or pGL3^{GPx8mut1}, or pGL3$^{GPx8\ mut2}$ and treated with 20mM DES or vehicle (control). Fold increase of luciferase activity was measured as in Fig. 4. In the insert, a proof of the efficacy of siRNA treatment is shown. HIFα subunits were measured by RTPCR as reported in Methods using the oligonucleotides indicated in Table 1. Significance by Student's t statistic: P< 0.001 HIF2α silenced cells transfected with pGL3^{GPx8wt} or pGL3$^{GPx8\ mut2}$ vs. corresponding cells silenced with HIF1α or scrambled nucleotides, and HIF2α silenced cells transfected with pGL3^{GPx8mut1} vs. corresponding cells transfected with scrambled nucleotides; P< 0.1 HIF2α silenced cells transfected with pGL3^{GPx8mut1} vs. corresponding cells silenced with HIF 1 alpha.

Fig. 8. h*GPx8* expression and promoter activity are increased by FGF treatment. In A) HeLa cells treated with FGF (0.1μg/ml for 10 min) or vehicle (V) and GPx8 mRNA analyzed and quantified as in Figure 2. In B) HeLa cells were transfected with pGL3^{PGKHRE} or pGL3^{GPx8wt} and treated with FGF or vehicle as described in Methods. Fold increase of luciferase activity was measured as indicated in Figure 3.

Fig. 9. GPx8 silencing amplifies the signaling cascade induced by FGF or insulin. HeLa cells stably expressing a siRNA targeted to GPx8 or stably expressing shRNA (control cells) were treated with 0.1 μg/ml FGF or 20 μg/ml insulin for 10 min. Untreated (U) cells were used as a control. Total cell lysates were probed (A) for P-ERK1/2, ERK by western blotting and quantified as described under Methods. (B) for P-AKT and HSP90. Quantification data are plotted on the right. The P-ERK or P-AKT signal relative to control cells was normalized to the ERK or HSP90 signal respectively. The blots are representative of five independent experiments with similar results.

Tab. 1. Synthetic oligonucleotides used in the study

PCR primers:	**Sequence (5'-> 3')**	**note**
*Hs*GPx8-fw	TGCAGCTTACCCGCTAAAAT	
*Hs*GPx8-rev	ATGACTTCAATGGGCTCCTC	
pGL3^{GPx8wt}-fw	AATA**GGTACC**ATTCATGACCGTTAGCAACA	KpnI restriction site bolfaced
pGL3^{GPx8wt}-rev	ATTA**AGATC**TGAAGTCTCAGCAGCCTGGAATTCA	BglII restriction sites bolfaced
pGL3^{GPx8mut1}-fw	ACGAAGCAATTAGATGA**TTAAT**CCAAGTAGAGGTGGGTTTTG	GPx8-HRE1 boldfaced
pGL3^{GPx8mut1}-rev	CAAAACCCACCTCTACTTGG**ATTAA**TCATCTAATTGCTTCGT	GPx8-HRE1 boldfaced
pGL3^{GPx8mut2}-fw	CAAAGTGCTGGGATTACAG**TTAAT**AGCCACCATGCCCGGCCTCAG	GPx8-HRE2 boldfaced
pGL3^{GPx8mut2}-rev	CTGAGGCCGGGCATGGTGGCT**ATTAA**CTGTAATCCCAGCACTTTG	GPx8-HRE2 boldfaced
*Hs*HIF1α-fw	AGTTGAATCAGAAGATAC	
*Hs*HIF1α-rev	TGAGTCTGCTGGAATACT	
*Hs*HIF2α-fw	AAGCATCCCTGCCACCGT	
*Hs*HIF2α-rev	ATCAAAGGGCAGCTCCCA	
HIFα subunits silencing:		
SiRNA-HIF1α-sense	GCCACUUCGAAGUAGUGCUdTdT	From ref. [33]
SiRNA-HIF2α-sense	GCGACAGCUGGAGUAUGAAdTdT	From ref. [33]
SiRNA-scramble-sense	UAGCGACUAAACACAUCAAUUdTdT	
EMSA analysis:		
GPx8-HRE1-fw,	GAAGCAATTAGATGACGTGCCCAAGTAGAG	
GPx8-HRE1-rev	CTCTACTTGGGCACGTCATCTAATTGCTTC	
GPx8-HRE2-fw	TGGGATTACAGGCGTGAGCCACCATGCCCG	
GPx8-HRE2-rev	CGGGCATGGTGGCTCACGCCTGTAATCCCA	
EPO-HREs-fw	ACGTGCTTACGTGCTTACGTGCTTACGTGC	
EPO-HREs-rev	GCACGTAAGCACGTAAGCACGTAAGCACGT	